The effects of various chemokines on vaccine induced responses were analyzed individually. Antisera from pCEnv and pCEnv + IL-8 immunized mice were collected and analyzed for specific antibody responses against HIV-1 gp120 protein by ELISA. The gp120-specific antibody titer from sera collected at weeks 0, 2,4, and 6 post-DNA immunization was measured. At 1:128 dilution, sera from the groups immunized with pCEnv+IL-8 showed antibody response against gp120 protein which was greater than that of the group immunized with pCEnv alone. A similar result was seen with the groups immunized with pCGag/pol. Furthermore, the subclasses of gp120-specific IgGs induced by the co-administration with IL-8 genes were determined. Production of IgG1 type is induced by Th2 type cytokines, whereas the IgG2a type production is induced by Th1 type cytokines. The relative ratios of IgG1 to IgG2a (Th2 to Th1) were measured. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, co-injection with pCEnv+IL-8 decreased the relative ratio to 0.9, indicating a shift to Th1-type response. IL-8 therefore influenced both the quality and quantity of the antigen-specific response.

The effect of IL-8 expression on T helper cell proliferative response was also examined. IL-8 co-expression with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in a dramatic level of antigen-specific T helper cell proliferative responses. The increase in proliferation was between 4 and 6 fold, a significant increase in antigen-specific responses. In addition, the effect of IL-8 co-expression on the induced CTL response was also investigated. A background level of specific killing was observed from the control animals, whereas the animals immunized with pCEnv alone showed a small, but consistent level of CTL response. IL-8 co-administration did not have any enhancement effect on the antigen-specific CTL response. Similar CTL results were observed from pCGag/pol+IL-8 co-immunization.

Cytokines play a key role in directing and targeting immune cells during immune response. For instance, IFN-γ is intricately involved in the regulation of T cell-mediated cytotoxic immune responses while IL-4 plays a dominant role in B cell-mediated immune responses. TNF-α is produced by activated macrophages and monocytes, neutrophils, activated lymphocytes, and NK cells and has been suggested to play a pivotal role in regulating the synthesis of other proinflammatory cytokines. We

analyzed supernatant from the effector cells stimulated *in vitro* for CTL assay and tested them for the release of cytokines IFN-γ, IL-4, and TNF-α. We found that IL-8 expression increased the level of IFN-γ only slightly, but it did not affect the levels of cytokines IL-4 and TNF-α. This is somewhat surprising as the dramatic effect of IL-8 co-delivery on the humoral responses might have been expected to have a noticeable effect on IL-4. However, this was not observed.

MIP1-α is a strong inducer of antibody response

MIP1-α co-expression exhibited a more drastic effect than IL-8 in the induction of antigen-specific humoral response. PCEnv+MIP1-α co-immunization resulted in a dramatic enhancement of envelope-specific antibody response. A similar result was seen with the groups immunized with pCGag/pol. The relative ratios of IgG1 to IgG2a following the co-administration with pCEnv+MIP1-α were determined. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, co-injection with pCEnv+MIP1-α decreased the relative ratio to 1.7, indicating a shift to Th2-type response. MIP1-α co-expression with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in enhancement of antigen-specific T helper cell proliferative responses. In contrast, MIP1-α immunization had minimal effect on the antigen-specific CTL responses or the induction of cytokines. Again, as was observed in the analysis of IL-8, effects on cytokine production, no effect was noted on IL-4 levels.

RANTES induces Th1 as well as CTL responses

We next examined the effects of RANTES co-delivery on vaccine induced immune responses. Unlike IL-8 or MIP1-α, co-expression of RANTES with pCEnv did not enhance HIV-1 envelope-specific antibody response. In addition, pCEnv-RANTES co-immunization did not have any effect on the IgG1-IgG2a ratio when compared to the group immunized with pCEnv alone. In contrast to the antibody responses, RANTES co-vaccinating with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in significant augmentation of antigen-specific T helper cell proliferative responses. Furthermore, two times higher level expressions of Th1 cytokines IFN-γ and TNF-α were observed from the group co-administered with pCEnv+RANTES. Unlike co-injection with PCEnv+IL-8 or pCEnv-MIP-1α which resulted in a minimal effect in CTL activity, a more dramatic increase in the specific killing of targets infected with

vaccinia (vMN462) expressing HIV-1 envelope was observed after co-injection with pCEnv+RANTES. Greater than 36% specific lysis of target cells was observed after co-injection with pCEnv+RANTES at a 50:1 effector to target (E:T) ratio. Similarly, the mice immunized with pCGag/pol+RANTES resulted in a significant enhancement of antigen-specific CTL lysis of targets infected with vaccinia (vVK1) expressing HIV-1 gag/pol. RANTES co-delivery appeared to polarize the resulting responses towards a Th1 type phenotype as no effect on IL-4 was again noted.

MCP-1 induces CTL responses

Adjuvant properties of MCP-1 cDNA was next observed. MCP-1 appeared to have a minimal effect on the specific antibody binding profile induced by pCEnv immunization. Moreover, MCP-1 co-expression with HIV-1 immunogens (pCEnv or pCGag/pol) had positive, but relatively minor (two fold) enhancement of antigen-specific T. Helper cell proliferative responses. The relative ratios of IgG1 to IgG2a following the co-administration with pCEnv+MCP-1 were determined. The pCEnv immunized group had a IgG1 to IgG2a ratio of 1.3. On the other hand, co-injection with pCEnv+MCP-1 decreased the relative ratio to 1.0, indicating a shift to Th1-type response. A more dramatic increase in the specific killing was observed after co-injection with pCEnv+MCP-1. Greater than 36% specific lysis of target cells was observed after co-injection with pCEnv+MCP-1 at a 50:1 effector to target (E:T) ratio. Similarly, the mice immunized with pCGag/pol_MCP-1 resulted in a significant enhancement of antigen-specific CTL lysis of HIV-1 gag/pol expressing targets. The level of IFN-γ release by mice immunized with pCEnv+MCP-1 was significantly greater than those of the pCEnv immunized or the control groups. Again, the level of IL-4 released from all groups were similar. Moreover, the level of TNF-α release by pCEnv+MCP-1 immunized group was significantly greater than those of the pCEnv immunized or the control groups. These cytokine release data support our CTL results which elucidate the roles of MCP-1 in the activation of $CD8^+$ CTL.

Determination of CD8 restriction in CTL response

To determine whether the increases in CTL response via co-expression on MCP-1 and RANTES was restricted to $CD8^+$ T cells, CTL assays were performed using a HIV-1 envelope peptide (RIHIGPGRAFYTTKN) which has been shown to be a

specific epitope for MHC class I-restricted CTL for balb/c mice. Mice received two immunizations of 50 μg of each DNA construct separated by two weeks and their spleens were harvested one week after the second immunization. The CTL assay was performed on the splenocytes following in vitro stimulation with envelope-specific peptides. We observed a significant enhancement of CTL response after both co-injection with MCP-1 and RANTES at 35% and 26% specific killing at an E:T ratio of 50:1, respectively. We verified this observation by measuring CTL activity after the removal of $CD8^+$ T cells from the effector cell population by complement lysis. The removal of $CD8^+$ T cells resulted in the suppression of antigen-specific CTL enhancement observed after co-injections with MCP-1 and RANTES. These results indicate that the enhancement of cytolytic activity was antigen-specific, class I-restricted and CD8 + T cell dependent.

Enhancement of chemokine expression

It was important to determine the effects, if any, of those specific chemokine adjuvanted immunogens on chemokine production itself. We examined the expression of chemokines MIP1-α, MIP1-β, RANTES, and MCP-1 by stimulated cells collected from immunized animals. Chemokine co-injection modulated chemokine production in chemokine specific patterns. We made several important observations. Furthermore, we observed that co-immunization with chemokine genes resulted in increased expression of chemokines by the stimulated cells. For instance, we observed that MIP1-α expression could be enhanced dramatically by co-immunization wit pCEnv + MIP1-α over the level expressed by pCEnv immunization alone. In addition, we found that MIP1-β expression was dramatically enhanced by pCEnv + MCP-1 and pCEnv + RANTES immunization, the two most significant inducers of CTL responses. Moreover, pCEnv + MIP1-α, pCEnv + MCP-1, and pCEnv + RANTES co-immunizations resulted in significant enhancement of RANTES expression by stimulated cells. Finally, the expression of MCP-1 was highest with pCEnv + MIP1-α and pCEnv + MCP-1 co-immunizations.

Discussion

The initiation of immune of inflammatory reactions is a complex process involving a tight coordinated expression of cellular adhesion molecules, cytokines, and chemokines. The chemokines are especially important in the molecular regulation of

trafficking of leukocytes from the vessels to the peripheral sites of host defense. The superfamily of chemokines consists of any array of over 20 related proteins. Chemokines are broadly divided into three families, C-X-C (α), C-C(β), and C(γ), based on the presence and position of the conserved cysteine residues. In the members of the α family, the first two cysteines are separated by another amino acid, while those of the β family are placed next to each other. Only two members of the γ family have been identified so far, and both of them contain one instead of two cysteines in their N-terminus.

Members of each subfamily have unique as well as overlapping activities. While exact physiological and pathological functions are not yet clearly defined, certain simplifying generalities can be made from the literature. It has been reported that, in general, the members of the C-X-C family are chemoattractants and activators for polymorphonuclear leukocytes including neutrophils, eosinophils, and basophils. In contrast, the members of the C-C family serve as chemotactic factors to mononuclear cells such as monocytes and lymphoctyes. On the other hand, the C-X-C chemokines, IL-8 and IP-10, which are reportedly chemotactic to T-lymphocytes and C-C chemokines, MCP-1, MPC-3, RANTES, and MIP-1α w which are also chemotactic to basophils. In general, the function of chemokines appears to be recruitment and activation of leukocytes at the site of inflammation.

In addition their functions in inflammatory and immune responses, some chemokines play a critical role in the transmission and progression of HIV-1 and 2 viruses responsible for AIDS. It has been anticipated for over a decade that binding of HIV envelope glycoprotein gp120 to CD4 is not sufficient for viral fusion and entry, suggesting the requirement for an additional cell-surface cofactor for HIV infection. Recent studies have identified that the co-receptors required for the fusion of the T cell-tropic and macrophage-tropic viruses with their target cells to be CXCR-4 and CCR-5, respectively.

CXCR-4, also known as fusion or LESTR, was originally discovered as an orphan receptor with structural similarity to chemokine receptors. CXCR-4 was subsequently identified as a necessary cofactor for entry of T cell-tropic HIV viruses into $CD4^+$ cells. The β-chemokine SDF-1 is a ligand for CXCR-4 and a powerful inhibitor of

infection by T cell-tropic HIV-1 strains. Similarly, the β-chemokines MIP-1α, MIP-1β, and RANTES are natural ligands for CCR-5 and are the major HIV suppressive factors produced by $CD8^+$ T cells for macrophage-tropic, but not T cell tropic, HIV isolates.

In these studies, a significant level of chemokine expression was observed following injection with a DNA immunogen. These results implied their potential roles as important activators and regulators of immune responses. To elucidate the specific roles of these chemokines in immune induction and modulation, we utilized the co-delivery of chemokine DNA expression cassettes as an antigen delivery model. DNA co-immunization is an appropriate model to investigate the in vivo functions of chemokines because DNA vaccines induce both humoral and cellular immune responses via both the MHC class I and II pathways. Furthermore, we and others have shown that antigen-specific immune responses to DNA vaccines can be modulated by the co-injection of costimulatory molecule and cytokine genes with DNA immunogen cassettes. Thus, we cloned and co-immunization chemokine expression vectors with HIV-1 DNA immunogens, and examined the effects of chemokine expression on immune activation. We observed that α-chemokine IL-8 and β-chemokines MIP-1α, RANTES, and MCP-1 had specific, identifiable roles in the activation of antigen-specific immune responses.

For instance, IL-8 is a chemotactic factor for neutrophils, inducing them to leave the bloodstream and migrate into the surrounding tissues. We observed that IL-8 was a strong inducer of $CD4^+$ T cells, indicated by strong T helper proliferative responses as well as the antibody responses. IL-8 co-expression also modulated the shift of immune responses to Th1-type, indicated by the reduction of IgG1 to IgG2a ratio and enhanced expression of IFN-γ. On the other hand, IL-8 co-administration did not have to noticeable effect on $CD8^+$ T cells, since it did not have any enhancement effect on the CTL response.

MIP-1α can chemoattractant and degranulate eosinophils. MIP-1α also induces histamine releases from basophils and mast cells, and is a chemotactic factor for basophils and B cells These reports support our observation that MIP-1α had the greatest effect on antibody responses. In addition, MIP-1α was also a strong inducer of $CD4^+$ T cells, with good T helper proliferative responses. MIP-1α co-expression also modulated the shift of immune responses to Th2-type, indicated by the increases of IgG1 to IgG2a

ratio. In contrast, MIP1-α co-immunization had minimal effect on the $CD8^+$ T cell response.

Unlike the effects of IL-8 and MIP-1α, RANTES co-immunization had minimal effect on antibody responses. RANTES is a monocyte chemoattractant. In addition, RANTES can chemoattract unstimulated $CD4^+/CD45RO^+$ memory T cells and stimulated $CD4^+$ and $CD8^+$ T cells. We observed that ability of RANTES to chemoattract $CD4^+$ and $CD8^+$ T cells to the site of DNA immunization served an important role in inducing T helper proliferative responses and CTL responses. The enhanced activation of Th1 responses was supported by the increased expression of Th1 cytokines IFN-γ and TNF-α. The high level of CTL responses induced by RANTES expression was determined to be class I-restricted and $CD8^+$ T cell dependent.

As a potent chemotactic factor for monocytes, MCP-1 is thought to be one of the most important chemokines for chronic inflammatory diseases. MCP-1 induces monocytes to migrate from the bloodstream to become tissue macrophages. MCP-1 was found to chemoattract T lymphocytes of the activated memory subset. Among all chemokines examined, MCP-1 is the most potent activator of CD8 + CTLs. The enhancement of CTL responses induced by MCP-1 expression was determined to be class 1-restricted and CD8 + T cell dependent. The enhanced CTL results are supported by increased expression to Th1 cytokines IFN-γ and TNF-α and the reduction of IgG11 to IgG2a ratio. Unlike RANTES, MCP-1 had positive, but moderate effect on the T helper cell proliferative responses. Like RANTES, MCP-1 co-administration had minimal effect on antibody responses. This comparison highlights that the induction of humoral, T helper, and T cytotoxic responses could be modulated independently of each other.

In addition to their direct effects on immune responses, co-expression of chemokine genes resulted in increased expression in autocrine manner. For instance, we observed that MIP1-α expression could be enhanced dramatically by co-immunization with pCEnv + MIP1-α over the level expressed by pCEnv immunization alone. Similar increases in RANTES was observed from RANTES co-delivery and MCP-1 increased.

Furthermore, co-expression of chemokine also resulted in enhanced expression of other chemokines. These results imply that these chemokines not only

have direct role in modulating immune responses, but they also act to control the production of other chemokines.

An important observation was the roles chemokine RANTES and MCP-1 play in inducing TNF-α expression. TNF-α is produced by activated macrophages and monocytes, neutrophils, activated lymphocytes, and NK cells whereas TNF-β is produced by lymphocytes. TNF-α is also implicated in septic shock following infection by Gram-negative bacteria and in rheumatoid arthritis. Furthermore, TNF-α plays a pivotal role in regulating the synthesis of other proinflammatory cytokines. Given TNF-α's critical roles in various ailments, there has been major efforts in reducing the level of TNF-α in vivo as potential treatment for conditions such as rheumatoid arthritis. In our experiments, we observed that co-expression of RANTES or MCP-1 resulted in the enhanced expression of TNF-α. These results imply that inhibiting RANTES and MCP-1 could compose a relevant strategy to curve TNF-α expression in vivo.

It is of interest that Th1 versus Th2 phenotype appears to segregate independently of other immune functions. IL-8 boosted humoral responses but drives those responses towards a Th1 phenotype, cutting the IgG1/IgG2a ratio in half. While MIP-1α, perhaps the most prolific driver of serology, skewed the IgG1/IgG2a ratio dramatically towards a Th2 response. It is clear that this manipulation can allow for induction of primary antigen-specific immune responses skewed towards a desired phenotype as well as immunoglobulin isotype independently of each other. Furthermore, the induction of cellular versus higher humoral responses appeared to be relatively polarized immune functions. Those chemokines with the most dramatic effect on humoral responses. IL-8 and MIP-1α, exhibited little effect on CTL responses whereas those which mediated the most dramatic effects on CTL responses, RANTES and MCP-1 had minimal effects on serology. The same CTL driving chemokines RANTES and MCP -1 both stimulated IFN-γ and TNF-α, while the humoral responders had minimal effects on these cytokine maker of immune activation.

Example 2

When the nucleotide sequence encoding IL-18 is delivered to certain cells as part of a vaccine or immunotherapeutic, it is less effect because it is inactive in full-

length form and only becomes active when processed in mature form. As shown in Figures 1A, the first 35 amino acids of IL-18 are cleaved by caspase-1 (ICE). A mutant IL-18 nucleotide sequence was constructed which is translated into the mutant IL-18 shown in Figure 1B. This mutant form of IL-18 operates as an effective immunomodulating protein according to the invention. Delivery of nucleotide sequences that encode the mutant IL-18 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response. The nucleotide sequence that encodes the mutant form may be inserted into pCDNA3.

Example 3

Delivery of nucleotide sequences that encode CD40 in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode CD40 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode CD40L in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode Fas in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode ICAM-1 in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode ICAM-1 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode LFA-3 in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode LFA-3 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode VCAM-1 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode PECAM-1 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode E-selectin in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode MC-SF in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode GC-SF in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode M-CSF in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode G-CSF in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode IL-4 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode E-selectin in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode IL-7 in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode NGF in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode VEGF in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode IL-7 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode NGF in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode VEGF in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode MCP-1 in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode RANTES in combination with nucleotide sequences that encode and immunogen result in an enhanced CTL response.

Delivery of nucleotide sequences that encode MCP-1 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response. Delivery of nucleotide sequences that encode RANTES in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of nucleotide sequences that encode MIP-1α in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response. Delivery of nucleotide sequences that encode IL-8 in combination with nucleotide sequences that encode and immunogen result in an enhanced helper T cell response.

Delivery of any one of MCP-1, RANTES, MIP-1α and IL-8 in combination with nucleotide sequences that encode and immunogen result in an enhanced antibody response.

Delivery of any one of MCP-1 or RANTES results in enhanced TNF-α production.

Delivery of any one of MCP-1, RANTES, MIP-1α and IL-8 in combination with nucleotide sequences that encode and immunogen result in enhanced IFN-γ production.

Example 4

We examined the effects on the antigen-specific immune responses following the co-delivery of the gene expression cassettes for M-CSF (macrophage-colony stimulating factor), G-CSF (Granulocyte-CSF), and GM-CSF (Granulocyte/Monocyte-CSF) along with HIV-1 DNA immunogen constructs. The genes for these cytokines were individually cloned into expression vectors under control of a cytomegalovirus (CMV) promoter. The gene plasmid expression cassettes were then injected into mice along with DNA vaccine cassettes for HIV-1 immunogens. We analyzed the immunological effects of the co-injection with these genetic adjuvant cassettes on the direction and magnitude of antigen-specific immune responses; these results were compared with the results we observed with co-delivery of genes for prototypical Th1 and Th2-type cytokines (IL-12 and IL-4, respectively). Using these DNA vaccine constructs as model antigens, we observed that CSF genes can dramatically and distinctly regulate antigen specific immune responses *in vivo*, and drive β-chemokine production in a vaccine-specific fashion.

Materials and Methods

DNA Plasmids

DNA vaccine constructs expressing HIV-1 envelope protein (pCEnv) and gag/pol protein (pCGag/Pol) were prepared as described in Boyer, J. D., et al. (1997) *Nature Med.* **3,** 526-532, which is incorporated herein by reference. The genes for human G-CSF and M-CSF as well as murine GM-CSF were cloned into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA) as described in Kim, J. J., et al. (1998) *Eur. J. Immunol.* **28,** 1089-1103 and Kim, J. J., et al. (1998) *J. Clin. Invest.* **102,** 1112-1124, which are incorporated herein by reference. Human G-CSF and M-CSF have been reported to be active in mouse cells. Clean plasmid DNA was produced in bacteria and purified using Qiagen Maxi Prep kits (Qiagen, Santa Clara, CA).

Reagents and cell lines

Mouse mastocytoma P815 cell lines were obtained from ATCC (Rockville, MD). Recombinant vaccinia expressing HIV-1 envelope (vMN462), gag/pol (vVK1), and β-galactosidase (vSC8) were obtained from the NIH AIDS Research and Reference Reagent Program. Recombinant gp120 or p24 protein were obtained from ImmunoDiagnostics, Inc. (Bedford, MA).

DNA inoculation of mice

The quadriceps muscles of 6 to 8 weeks old female BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with 50 μg of each DNA construct of interest formulated in phosphate buffered saline (PBS) and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO). Co-administration of various gene expression cassettes involved mixing the chosen plasmids prior to injection. The control mice were immunized with 50 μg of pCDNA3 vector. Each set of studies was performed three times and a representative set of results is presented. Mice received two DNA immunization (50 mg each) separated by two weeks. At one week after the boost injection, the mice were sacrificed, the spleens were harvested, and the lymphocytes were isolated and tested for cellular (Th or CTL) responses. All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was under the guidelines of the National Institute of Health and the University of Pennsylvania.

ELISA

Fifty μl of p24 or gp120 protein diluted in 0.1M carbonate-bicarbonate buffer (pH 9.5) to 2 mg/ml concentration was adsorbed onto microtiter wells overnight at 4 °C. The plate were washed with PBS-0.05% Tween-20 and blocked with 3% BSA in PBS with 0.05% Tween-20 for one hour at 37°C. Mouse antisera was diluted with 0.05% Tween-20 and incubated for one hour at 37°C, then incubated with HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The plates were washed and developed with 3'3'5'5' TMB (Sigma) buffer solution. For the determination of relative levels of gp120-specific IgG subclasses, anti-murine IgG1 and IgG2a conjugated with HRP (Zymed, San Francisco, CA) was substituted for anti-murine IgG-HRP. This was followed by addition of the ABTS substrate solution (Chemicon, Temecula, CA). In each step, plates were washed 3 times with the wash buffer (PBS + 0.05% Tween-X).

The plates were read on a Dynatech MR5000 plate reader with the optical density at 450 nm.

T helper cell proliferation assay

Lymphocytes were harvested from spleens and prepared as the effector cells by removing the erythrocytes and by washing several times with fresh media. The isolated cell suspensions were resuspended to a concentration of 5 x 10^6 cells/ml. A 100 µl aliquot containing 5 x 10^5 cells was immediately added to each well of a 96 well microtiter flat bottom plate. Recombinant p24 or gp120 protein at the final concentration of 5 µg/ml and 1 µg/ml was added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for three days. One mCi of tritiated thymidine was added to each well and the cells were incubated for 12 to 18 hours at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). Stimulation Index was determined from the formula:

Stimulation Index (SI) = (experimental count/ spontaneous count)

Spontaneous count wells include 10% fetal calf serum which serves as irrelevant protein control.

Cytotoxic T lymphocyte assay

A five hour ^{51}Cr release CTL assay was performed using vaccinia infected targets. The assay was performed with *in vitro* effector stimulation, where the effectors were stimulated with relevant vaccinia-infected cells (vMN462 for envelope and vVK1 for gag/pol) which were fixed with 0.1% glutaraldehyde for five days in CTL culture media at 5 x 10^6 cells/ml. The effectors were stimulated non-specifically for two days with CTL culture media consisting of RPMI 1640 (Gibco-BRL, Grand Island, NY), 10% fetal calf serum (Gibco-BRL) and 10% RAT-T-STIM without Con A (Becton Dickinson Labware, Bedford, MA). Vaccinia infected targets were prepared by infecting 3 x 10^6 P815 cells at the multiplicity of infection (MOI) of 10-20 for five to twelve hours at 37°C. A standard Chromium release assay was performed in which the target cells were labeled with 100 mCi/ml $Na_2{}^{51}CrO_2$ for 60 to 120 minutes and used to incubate with the stimulated effector splenocytes for four to six hours at 37°C. CTL lysis was determined at effector:target (E:T) ratios ranging from 50:1 to 12.5:1. Supernatants were harvested

and counted on a LKB CliniGamma gamma-counter. Percent specific lysis was determined from the formula:

$$100 \times \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}}$$

Maximum release was determined by lysis of target cells in 1% Triton X-100 containing medium. An assay was not considered valid if the value for the 'spontaneous release' counts is in excess of 20% of the 'maximum release'.

Complement lysis of CD8+ T cells

$CD8^+$ T cells were removed from the splenocytes by a treatment with α-CD8 monoclonal antibody (Pharmingen, San Diego, CA) followed by incubation with rabbit complement (Sigma) for 45 min. at 37 °C.

Cytokine/chemokine expression analysis

Supernatants from effectors stimulated for CTL assay were collected at day 6 and tested for cytokine and chemokine profiles using ELISA kits for IFN-γ and IL-4 (Biosource International, Inc., Camarillo, CA), and MIP-1α (R&D Systems, Minneapolis, MN), MIP-1β, and RANTES (Intergen, Pace, NY).

RESULTS

Construction of cytokine expression cassettes

The cytokine genes were individually cloned into pCDNA3 plasmid expression vectors. To test whether the cytokine constructs expressed their relevant proteins, we transfected them *in vitro* into RD muscle cell line, and the expression of these constructs were analyzed by cytokine ELISA. Results demonstrate that each expression cassettes produced specific cytokines (G-CSF ~40-60 pg/ml; GM-CSF >70pg/ml; M-CSF ~60-70 pg/ml).

G-CSF induces enhancement of T helper response

G-CSF is a growth factor produced by macrophages, fibroblasts, endothelial cells, and bone marrow stromal cells. G-CSF activates neutrophils, endothelial cells, and platelets, but is thought to have little direct effects on antigen presenting cells. We examined the effects of G-CSF co-expression on antigen specific antibody responses. Antisera from pCEnv and pCEnv+G-CSF immunized mice were collected and analyzed

for specific antibody responses against HIV-1 gp120 protein by ELISA. The gp120-specific antibody titer from sera collected at 6 weeks post-DNA immunization was measured. G-CSF co-immunization did not significantly affect the level of gp120-specific antibody response. A similar result was observed with the groups immunized with pCGag/pol. Furthermore, the subclasses of gp120-specific IgGs induced by the co-administration with G-CSF genes were determined. It has been reported that production of IgG1 type is induced by Th2 type cytokines, whereas the IgG2a type production is induced by Th1 type cytokines. The relative ratios of IgG2a to IgG1 (Th1 to Th2) were measured. The pCEnv immunized group had a IgG2a to IgG1 ratio of 0.8. On the other hand, co-immunization of prototypical Th1 cytokine IL-12 genes increased the ratio to 1.28 and while co-injection with Th2 cytokine IL-4 gene resulted in a reduction of the ratio to 0.68. Co-administration with G-CSF increased the relative ratio to 1.1, indicating a shift to Th1-type response.

The effect of G-CSF co-expression on T helper cell proliferative response was also examined. T helper lymphocytes play a critical role in inducing both a humoral immune response via B cells and cellular immune response via $CD8^+$ cytotoxic T cells. Co-immunization of IL-12 genes dramatically enhanced the level of antigen-specific Th proliferative responses. In contrast, co-injection with IL-4 gene had minimal effects on the Th proliferative responses. G-CSF co-expression with HIV-1 immunogens resulted in positive enhancement of antigen-specific T helper cell proliferative responses.

In addition, the effects of cytokine co-expression on CTL response were also investigated. A background level of specific killing was observed from the control animals, whereas the animals immunized with pCEnv or pCGag/pol showed a small, but positive level of antigen-specific CTL responses. Co-injection with IL-12 genes dramatically enhanced the level of antigen-specific CTL responses. In contrast, co-immunization with IL-4 genes had minimal effects on the responses. Similarly, G-CSF co-administration did not have any enhancement effect on antigen-specific CTL responses.

Cytokines play a key role in directing and targeting immune cells during immune response. For instance, IFN-γ is intricately involved in the regulation of T cell-mediated cytotoxic immune responses while IL-4 plays a dominant role in B cell-mediated

immune responses. We analyzed supernatant from the effector cells stimulated *in vitro* for CTL assay and tested them for the release of cytokines IFN-γ and IL-4. We found that G-CSF expression increased the level of IFN-g (2-fold), but it did not affect the level of IL-4 production.

GM-CSF is a potent inducer of both antibody and T helper responses

GM-CSF activates and differentiates granulocytic cells and can serve as growth factor for endothelial cells, erythroid cells, megakaryocytes, and T helper cells. It is unclear if GM-CSF can have effects on killer T cells. In contrast to G-CSF co-expression, GM-CSF co-expression had a significant enhancement effect (highest in all groups) in the induction of antigen-specific humoral response. Similar to IL-4 co-injection, GM-CSF co-immunization resulted in the highest level of envelope-specific antibody response. A similar result was seen with the groups immunized with pCGag/pol. On the other hand, pCEnv+GM-CSF co-immunization did not have any effect on the IgG2a/IgG1 ratio when compared to the group immunized with pCEnv alone. Furthermore, along with IL-12 co-immunization, GM-CSF co-expression with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in the highest level of antigen-specific T helper cell proliferative responses. We also found that GM-CSF expression increased the level of IFN-γ (2-fold), but it did not affect the level of IL-4 production. In contrast, GM-CSF co-immunization had only slight effect on the antigen-specific CTL response.

M-CSF is a potent inducer of CTL response

M-CSF is a potent activator of macrophages as well as macrophage progenitor cells. The M-CSF receptor has a restricted expression pattern, again limited to macrophages. As such the direct effects of M-CSF co-delivery on this APC population can be evaluated. Unlike GM-CSF, co-expression of M-CSF with pCEnv had positive enhancement effect (but less than IL-4 or GM-CSF) on the HIV-1 envelope-specific antibody response. The relative ratios of IgG2a to IgG1 following the co-administration with pCEnv+M-CSF were determined. The pCEnv immunized group had a IgG2a to IgG1 ratio of 0.8. On the other hand, co-injection with pCEnv+M-CSF increased the relative ratio to 1.2, indicating a strong shift to Th1-type response. Furthermore, M-CSF co-expression with HIV-1 immunogens (pCEnv or pCGag/pol) resulted in significant

augmentation of antigen-specific T helper cell proliferative responses. Unlike co-injection with pCEnv+G-CSF or pCEnv+GM-CSF which resulted in a minimal effect in CTL activity, a more dramatic increase in the specific killing of targets infected with vaccinia (vMN462) expressing HIV-1 envelope was observed after co-injection with pCEnv+M-CSF. Almost 40% specific lysis of target cells was observed after co-injection with pCEnv+M-CSF at a 50:1 effector to target (E:T) ratio. Similarly, the mice immunized with pCGag/pol+M-CSF resulted in a significant enhancement of antigen-specific CTL lysis of targets infected with vaccinia (vVK1) expressing HIV-1 gag/pol. The level of IFN-γ release by mice immunized with pCEnv+M-CSF was significantly greater than those of the pCEnv immunized or the control groups. On the other hand, the level of IL-4 from these groups were similar.

Enhancement of CTL responses by M-CSF co-immunization is CD8 T cell-restricted

To determine whether the increase in CTL response via co-expression of M-CSF was restricted to $CD8^+$ T cells, CTL assays were performed using a HIV-1 envelope peptide (RIHIGPGRAFYTTKN) which has been shown to be a specific epitope for MHC class I-restricted CTL for balb/c mice. Mice received two immunizations of 50 μg of each DNA construct separated by two weeks and their spleens were harvested one week after the second immunization. The CTL assay was performed on isolated splenocytes following *in vitro* stimulation with envelope-specific peptides as described. We observed a significant enhancement of CTL response after both co-injection with M-CSF at 40% specific killing at an E:T ratio of 50:1. We verified this observation by measuring CTL activity after the removal of CD8+ T cells from the effector cell population by complement lysis. The removal of $CD8^+$ T cells resulted in the suppression of antigen-specific CTL enhancement observed after co-injections with M-CSF. These results indicate that the enhancement of cytolytic activity was antigen-specific, class I-restricted and $CD8^+$ T cell dependent.

Co-delivery of M-CSF genes modulate β-chemokine production by stimulated T cells

We examined the expression profiles of β-chemokines (MIP-1α, MIP-1β, and RANTES) from stimulated T cells. These β-chemokines are the major HIV suppressive factors produced by $CD8^+$ T cells for macrophage-tropic, but not T cell tropic, viruses. Moreover, these $CD8^+$ T cell-produced chemokines have been shown play a critical role

in cellular immune expansion in the periphery. Specifically, we observed that DNA immunization with pCEnv induces the β-chemokines MIP-1α, MIP-1β, and RANTES. Furthermore, we observed that co-immunization with hematopoietic cytokine genes resulted in increased expression of chemokines by the stimulated T cells. For instance, we observed that MIP-1α expression could be enhanced dramatically by co-immunization with pCEnv+G-CSF over the level expressed by pCEnv immunization alone. In addition, we found that MIP-1β expression was dramatically enhanced by pCEnv+M-CSF co-immunization. On the other hand, pCEnv+M-CSF, pCEnv+G-CSF, and pCEnv+GM-CSF co-immunizations did not result in significant enhancement of RANTES expression by stimulated effector cells over that induced by the DNA vaccine cassettes alone. Interestingly, however, M-CSF appears to down-modulate MIP-1α levels even lower than these induced by gene immunization on its own. This data could suggest that MIP-1α is not directly involved in driving a CTL response and may actually interfere with its induction.

DISCUSSION

The manipulation of the local immune environment, possibly in the periphery of regional lymph node at the injected site or in the muscle, may influence both the magnitude and direction of the immune response. We examined the immune effects derived from co-delivering genes for G-CSF, GM-CSF, and M-CSF as molecular adjuvants for DNA vaccines.

We observed that G-CSF, GM-CSF, and M-CSF cDNA constructs all modulated DNA vaccine's immune profile uniquely. G-CSF is a pleiotropic cytokine best known for its specific effects on the proliferation, differentiation, and activation of hematopoietic cells of the neutrophilic granulocyte lineage. It is produced mainly by monocytes and macrophages upon activation by a variety of stimuli including, endotoxins, IL-1, TNF-α, and IFN-γ. It regulates proliferation and maturation of neutrophilic granulocyte precursors and acts directly on mature neutrophils to enhance phagocytosis, ADCC, superoxide generation, chemotaxis, and expression of cell-surface adhesion molecules. *In vitro* administration of G-CSF can stimulate neutrophilic colony formation from bone marrow hematopoietic progenitor cells. Clinically, G-CSF is most commonly administered for the treatment of chemotherapy and radiation therapy-induced

neutropenia. We found that G-CSF co-immunization had minimal effect overall on antibody responses. G-CSF co-expression modulated the shift of immune responses to Th1-type, indicated by the increase of IgG2a/IgG1 ratio and enhanced expression of IFN-γ. Moreover, G-CSF co-expression resulted in a moderate enhancement of T helper proliferative responses. Overall G-CSF, which should not directly affect antigen presentation, had at best a moderate effect on antigen specific immune responses.

GM-CSF is a pleiotropic cytokine that can stimulate the proliferation, maturation, and function of a variety of hematopoietic cells. GM-CSF was first recognized for its ability to stimulate neutrophil, monocyte/macrophage, and eosinophil colony formation. It is produced by a variety of cell types, including T cells, B cells, macrophages, mast cells, endothelial cells, and fibroblasts, in response to cytokine or immune and inflammatory stimuli. We observed that GM-CSF was a strong inducer of CD4+ Th cells, indicated by strong T helper proliferative responses as well as strong boosting of antibody responses. On the other hand, pCEnv+GM-CSF co-immunization did not have any effect on the IgG2a/IgG1 ratio. In addition, GM-CSF co-administration did not seem to have noticeable effect on $CD8^+$ T cells, demonstrated by lack of any effects on the induction of antigen-specific CTL responses. Thus, vaccine help driven by this cytokine was entirely focused on the T helper cell. These results support and extend the previous studies on the use of GM-CSF cDNA constructs as a molecular adjuvant. It has been reported that intramuscular co-inoculation of plasmid expressing rabies virus glycoprotein and plasmid encoding mouse GM-CSF enhanced the B and T helper cell activity. Similarly, we reported that co-immunization of GM-CSF cDNA with DNA vaccine constructs increases antigen-specific antibody and Th-cell proliferation responses. In contrast, almost no effect on CTL induction was observed with the genes for GM-CSF in these studies. A similar finding was reported using GM-CSF co-delivery with DNA immunogen encoding for influenza nucleoprotein (NP). We also co-delivered GM-CSF cDNA constructs with DNA expression construct encoding for HSV-2 gD protein. We then analyzed the vaccine modulatory effects on resulting immune phenotype and on the mortality and the morbidity of the immunized animals following HSV lethal challenge. We observed that GM-CSF gene co-administration not only

enhanced survival rate, but also reduced the frequency and severity of herpetic lesions following intravaginal HSV challenge.

M-CSF has been shown to be the regulator of the growth, differentiation and function of mononuclear phagocytes. It also increases the expression of adhesion molecules and Fc receptors, increases tumoricidal activity, enhances secondary release of cytokines including IL-1, TNF-α, and IFN-γ. M-CSF was originally discovered in serum, urine and other biological fluids as a factor that could stimulate the formation of macrophage colonies from bone marrow hematopoietic progenitor cells. M-CSF can be produced by a number of cells, including fibroblasts, secretory epithelial cells of the endometrium, bone marrow stromal cells, brain astrocytes, osteoblasts, renal mesangial cells, keratinocytes and LPS or cytokine-activated macrophages, B cells, T cells and endothelial cells. Among the CSFs examined, M-CSF was the most potent activator of $CD8^+$ CTLs. The enhancement of CTL responses induced by M-CSF expression was both MHC class I-restricted and $CD8^+$ T cell dependent. The enhanced CTL results are supported by increased production of IFN-γ and the increased IgG2a/IgG1 ratio. Unlike the effects of GM-CSF, M-CSF co-immunization had weak effect on antibody responses. Similar to the effects of GM-CSF, M-CSF co-expression with HIV-1 immunogens resulted in augmentation of antigen-specific T helper cell proliferative responses, although to a lesser degree. It appears that the CTL pathway is particularly benefitted by this cytokine.

It was interesting to compare the immunomodulatory effects of Th1/Th2-type cytokine and CSF genes. For instance, GM-CSF co-injection positively modulated the level of antibody responses similar to IL-4 co-immunization. On the other hand, the IL-4 co-immunization led to a more Th2-type response as seen by dramatic reduction in IgG2a/IgG1 ratio. Furthermore, the dramatic enhancement effect of GM-CSF co-injection on the Th proliferative responses was only matched by that of IL-12 co-administration. On the other hand, co-delivery of gene for M-CSF, and not G-CSF or GM-CSF, resulted in a significant enhancement of $CD8^+$ T cell-restricted CTL responses. These results indicate that the production of these growth factors at the site of immune activation could regulate the level of DNA vaccine-induced immune responses in vivo. Moreover, these results imply that these growth factors could play a more active role in

modulating immune response cascade, which have been previously thought to be under the domain of traditional Th1 and Th2- type cytokines.

In addition to the analysis of the cell-based responses, we also examined the modulation of chemokine production resulting from the co-expression of CSF genes. Chemokines are important modulators of immune and inflammatory responses. They are especially important in the molecular regulation of trafficking of leukocytes from the vessels to the peripheral sites of host defense. Moreover, some chemokines have been shown play a critical role in the regulation on immune expansion in the periphery. We observed that $CD8^+$ effector T cells elevated chemokine expression levels while they primed immune responses suggests a regulatory role for these end-stage effector cells in the expansion phase of an antigen-specific immune response. In addition to their functions in inflammatory and immune responses, some chemokines may play a critical role in the transmission and progression of AIDS. It has been anticipated for over a decade that binding of HIV envelope glycoprotein gp120 to CD4 is not sufficient for viral fusion and entry, suggesting the requirement for an additional cell-surface cofactor for HIV infection. Recent studies have identified that the co-receptors required for the fusion of the T cell-tropic and macrophage-tropic HIV strains with their target cells to be CXCR-4 and CCR-5, respectively. The β-chemokines MIP-1α, MIP-1β, and RANTES are natural ligands for CCR-5 and are among the major HIV suppressive factors produced by $CD8^+$ T cells for macrophage-tropic, but not T cell tropic, HIV isolates.

We examined the expression of MIP-1α, MIP-1β, and RANTES by in vivo vaccine stimulated cells and observed that co-immunization with CSF genes resulted in increased expression of chemokines by antigen stimulated T cells. We observed that MIP-1α production could be dramatically enhanced by co-immunization with pCEnv+G-CSF over the level produced by pCEnv immunization alone. In particular, we found that MIP-1β expression was dramatically enhanced by pCEnv+M-CSF co-immunization. In contrast, none of the co-injection strategies resulted in significant enhancement of RANTES expression by stimulated effector cells. One other novel effect observed was the dramatic rise in RANTES and MIP-1β and at the same time, the decline in MIP-1α. In fact, MIP-1α levels induced by M-CSF were actually lower than control levels. This data suggests that MIP-1α signaling through CCR1, CCR4, and CCR5 results in a

different signals than that delivered by RANTES or MIP-1β through either CCR3 or CCR9. This finding might distinguish these putative receptor effects on the immune responses.

These results show that co-delivery of the growth factor cDNA constructs modulate both humoral and cellular (including the production of chemokines) immune responses. In particular, co-injection with M-CSF had most modulatory effect on antigen-specific CTL induction and chemokine production.

Example 5: ICAM-1 provides T cell costimulation and chemokine production

We examined the immunomodulatory effects of three specific adhesion molecules which are closely regulated by chemokines. We utilized DNA vaccine technology to explore the role of ICAM-1, LFA-3, and VCAM-1 in immune activation *in vivo*. Specifically, we cloned genes for ICAM-1, LFA-3, and VCAM-1 individually into expression vectors under control of a cytomegalovirus (CMV) promoter. These constructs were then co-immunized along with DNA immunogens encoding for HIV-1 envelope or gag/pol antigens. We analyzed the immunological effects of the co-injection with these adhesion molecule cassettes on the level of antigen-specific immune responses.

We observed that antigen-specific T cells responses can be enhanced by the co-expression of DNA immunogen and adhesion molecules ICAM-1 and LFA-3. However, ICAM-1 and LFA-3 appeared to play no role in expression of antigen-specific humoral responses. Rather, they appeared to specifically affect T cells responses. LFA-3 enhanced $CD4^+$ T cell responses and exhibited more minor effect on $CD8^+$ T cell function. More importantly, ICAM-1 co-administration dramatically increased both $CD4^+$ and $CD8^+$ T cell responses. ICAM-1 co-expression also dramatically enhanced antigen-specific β-chemokine production suggesting an important role for ligation of LFA-1 in peripheral T cell expansion. The activation phenotype of these molecules appeared to be distinct from the prototypic CD80/CD86 costimulatory molecules. These results support that the peripheral network of cytokine, chemokine, and adhesion molecules coordinately regulate effector T cell responses at the site of effector function.

Materials And Methods

DNA Plasmids

DNA vaccine constructs expressing HIV-1 envelope protein (pCEnv) and gag/pol protein (pCGag/Pol) were prepared as described in Kim, J. J., et al *Nature Biot.* 15:641-645, which is incorporated herein by reference. The genes for ICAM-1, LFA-3, and VCAM-1 were cloned into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA) and clean plasmid DNA was produced as described in Kim, J. J., et al. *Eur. J. Immunol.* 28:1089-1103.

Reagents and cell lines

Human rhabdomyosarcoma (RD) and mouse mastocytoma P815 cell lines were obtained from ATCC (Rockville, MD). Recombinant vaccinia expressing HIV-1 envelope (vMN462), gag/pol (vVK1), and β-galactosidase (vSC8) were obtained from the NIH AIDS Research and Reference Reagent Program. Recombinant gp120 or p24 protein were obtained from ImmunoDiagnostics, Inc. (Bedford, MA).

Expression of adhesion molecule expression constructs

Expression of ICAM-1, LFA-3, VCAM-1 constructs were analyzed by transfecting them into RD cells. Cells were harvested 72 hours after transfection and tested for expression using FACS analysis with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for ICAM-1, LFA-3, VCAM-1 (Pharmingen, San Diego, CA).

DNA inoculation of mice

The quadriceps muscles of 6 to 8 weeks old female BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with 50 μg of each DNA construct of interest formulated in phosphate buffered saline (PBS) and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO). Co-administration of various gene expression cassettes involved mixing the chosen plasmids prior to injection. The control mice were immunized with 50 μg of pCDNA3 vector. Each set of studies was performed three times and a representative set of results is presented. Mice received two DNA immunization (50 μg each) separated by two weeks. At one week after the boost injection, the mice were sacrificed, the spleens were harvested, and the lymphocytes were isolated and tested for cellular (Th or CT L (cytotoxic T lymphocyte)) responses.

ELISA

Fifty µg of p24 or gp120 protein diluted in 0.1M carbonate-bicarbonate buffer (pH 9.5) to 2 µg/ml concentration was adsorbed onto microtiter wells overnight at 4°C. The plate were washed with PBS-0.05% Tween-20 and blocked with 3% BSA in PBS with 0.05% Tween-20 for one hour at 37°C. Mouse antisera was diluted with 0.05% Tween-20 and incubated for one hour at 37°C, then incubated with HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The plates were washed and developed with 3'3'5'5' TMB (Sigma) buffer solution. The plates were read on a Dynatech MR5000 plate reader with the optical density at 450 nm.

T helper cell proliferation assay

Lymphocytes were harvested from spleens and prepared as the effector cells by removing the erythrocytes and by washing several times with fresh media. The isolated cell suspensions were resuspended to a concentration of 5×10^6 cells/ml. A 100 ml aliquot containing 5×10^5 cells was immediately added to each well of a 96 well microtiter flat bottom plate. Recombinant p24 or gp120 protein at the final concentration of 5 µg/ml and 1 µg/ml was added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for three days. One mCi of tritiated thymidine was added to each well and the cells were incubated for 12 to 18 hours at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). Stimulation Index was determined from the formula:

Stimulation Index (SI) = (experimental count/ spontaneous count)

Spontaneous count wells include 10% fetal calf serum which serves as an irrelevant protein control. In addition, pCEnv or control immunized animals routinely have SI of 1 against Pr55 protein. Similarly, pCGag/pol or control routinely have SI of 1 against gp120 protein. To assure that cells were healthy, PHA or con A (Sigma) was used as a polyclonal stimulator positive control. The PHA or con A control samples had a SI of 20-40.

Cytotoxic T lymphocyte assay

A five hour ^{51}Cr release CTL assay was performed using vaccinia infected targets. The assay was performed with *in vitro* effector stimulation, where the effectors

were stimulated with relevant vaccinia-infected cells (vMN462 for envelope and vVK1 for gag/pol) which were fixed with 0.1% glutaraldehyde for five days in CTL culture media at 5 x 10^6 cells per ml. The effectors were stimulated non-specifically for two days with CTL culture media consisting of RPMI 1640 (Gibco-BRL, Grand Island, NY), 10% fetal calf serum (Gibco-BRL) and 10% RAT-T-STIM without Con A (Becton Dickinson Labware, Bedford, MA). Vaccinia infected targets were prepared by infecting 3 x 10^6 P815 cells at the multiplicity of infection (MOI) of 10-20 for five to twelve hours at 37°C. A standard Chromium release assay was performed in which the target cells were labeled with 100 mCi/ml $Na_2{}^{51}CrO_4$ for 60 to 120 minutes and used to incubate with the stimulated effector splenocytes for four to six hours at 37°C. CTL lysis was determined at effector:target (E:T) ratios ranging from 50:1 to 12.5:1. Supernatants were harvested and counted on a LKB CliniGamma gamma-counter. Percent specific lysis was determined from the formula:

$$100 \times \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}}$$

Maximum release was determined by lysis of target cells in 1% Triton X-100 containing medium. An assay was not considered valid if the value for the 'spontaneous release' counts is in excess of 20% of the 'maximum release'.

Complement lysis of CD8+ T cells

$CD8^+$ T cells were removed from the splenocytes by a treatment with α-CD8 monoclonal antibody (Pharmingen, San Diego, CA) followed by incubation with rabbit complement (Sigma) for 45 min. at 37°C.

Cytokine and chemokine expression analysis

Supernatants from effectors stimulated for CTL assay were collected at day 6 and tested for expression using ELISA kits for IFN-γ and IL-4 and for MIP-1α, MIP-1β, and RANTES (Biosource, Camarillo, CA; R&D Systems, Minneapolis, MN; Intergen, Purchase, NY).

Results

ICAM-1, LFA-3, and VCAM-1 can be expressed by transfected cells

The genes for ICAM-1 (pCICAM-1), LFA-3 (pCLFA-3), and VCAM-1 (pCVCAM-1) were individually cloned into the pCDNA3 expression vector (Figure 2). To test whether pCICAM-1, pCLFA-3, and pCVCAM-1 constructs could express their relevant proteins, we transfected them *in vitro* into the human rhabdomyosarcoma (RD) cells. Using FACS analysis we observed that transfection of pCICAM-1, pCLFA-3, and pCVCAM-1 expression cassettes resulted in specific expression of ICAM-1, LFA-3, and VCAM-1, respectively. We also observed that co-immunization of two DNA expression cassettes intramuscularly resulted in co-expression of both encoded proteins in same muscle cells *in vivo*.

Co-expression of adhesion molecules does not affect Ag-specific humoral immune responses

We next investigated the effects the co-expression of adhesion molecules have on the induction of antigen-specific immune responses. For all experiments, 50 µg of each DNA expression constructs were injected into BALB/c mice intramuscularly at weeks 0 and 2. The first immune parameter examined was the antigen-specific humoral response. Antisera from immunized mice were collected at weeks 0, 2 and 6 and were analyzed for specific antibody responses against HIV-1 gp120 protein by ELISA. Co-expression of ICAM-1, LFA-3, or VCAM-1 appeared to have a minimal effect on the specific antibody binding profile induced by pCEnv immunization. A similar result was seen with the groups co-immunized with pCGag/pol.

Co-expression of ICAM-1 or LFA-3 enhances Ag-specific Th proliferative responses

The effect of adhesion molecule co-expression on the magnitude of cellular immune responses were also investigated. Induction of $CD4^+$ T helper cell proliferative response is important because Th cells play a critical role in inducing both a humoral immune response via B cells and CTL response via $CD8^+$ T cells. The Th proliferative responses for the mice immunized with pCGag/pol and those mice co-immunized with pCICAM-1, pCLFA-3, or pCVCAM-1 were measured. Recombinant gp120 HIV-1 envelope protein (5 µg/ml and 1 µg/ml) was plated in each well for specific stimulation of T cell proliferation. We also analyzed these groups for non-specific stimulation of T-cells using irrelevant proteins and observed that nonspecific antigen did not induce T-cell proliferative responses *in vitro*. A background level of proliferation was observed in the

control group immunized with a control vector, and a moderate level of proliferation was observed in the group immunized with pCEnv alone. In contrast, the groups co-immunized with either pCICAM-1 or pCLFA-3 had significantly higher levels of proliferative responses. On the other hand, the group co-immunized with VCAM-1 genes did not show any enhancement of antigen-specific Th response. A similar result was seen with the groups co-immunized with pCGag/pol. In repeat experiments using either immunogens, co-delivery of pCICAM-1 or pCLFA-3 resulted in a 3 to 4 fold increase in antigen-specific proliferative responses.

Co-expression of ICAM-1 or LFA-3 enhances Ag-specific CTL responses

To further investigate the enhancement of cellular immunity, we performed CTL assays using splenocytes of mice co-immunized with pCEnv and pCGag/pol. The assay was performed with *in vitro* stimulation of effector splenocytes prior to measuring chromium release from specific and non-specific vaccinia infected or peptide treated targets. To calculate specific lysis of targets, the percent lysis of irrelevant targets was subtracted from the percent lysis of specific targets. A background level of specific killing was observed from the control animals with pCDNA3, pCICAM-1, pCLFA-3, or pCVCAM-1 immunizations, while the animals immunized with pCEnv showed low level of CTL response. On the other hand, co-immunization with pCEnv+pCICAM-1 resulted in a dramatic increase in CTL activity. Greater than 40% specific killing of HIV-1 envelope vaccinia (vMN462) infected targets was observed after co-immunization with pCEnv+pCICAM-1 at a 50:1 effector to target (E:T) ratio. The CTL activity titered out to 20% specific lysis at a 12.5:1 E:T ratio. In contrast, co-immunization with pCEnv+pCLFA-3 resulted in a more moderate increase in CTL activity. Similar CTL results were observed following co-immunizations with pCGag/pol+pCICAM-1 and pCGag/pol+pCLFA-3.

To determine whether the increases in CTL response via co-expression of pCICAM-1 and pCLFA-3 were restricted to $CD8^+$ T cells, CTL assays were performed by measuring CTL activity with and without the removal of $CD8^+$ T cells from the effector cell population by complement lysis. The removal of $CD8^+$ T cells resulted in the suppression of antigen-specific CTL enhancement observed after co-injections with

pCICAM-1 and pCLFA-3. These results indicate that the enhancement of cytolytic activity was antigen-specific and $CD8^+$ T cell dependent.

Co-expression of ICAM-1 or LFA-3 increases production of IFN-γ by stimulated T cells

Analysis of cytokine production by stimulated CTLs in the immunized animals support the CTL results observed. Cytokines play a key role in directing and targeting immune cells during the development of the immune response. For instance, IFN-γ is intricately involved in the regulation of T cell-mediated cytotoxic immune responses while IL-4 plays a dominant role in B cell-mediated immune responses. We analyzed supernatant from the effector cells stimulated *in vitro* for CTL assay and tested them for the release of cytokines IFN-γ and IL-4. We found that co-injection with pCICAM-1 increased the level of IFN-γ significantly. Co-immunization with pCLFA-3 resulted in a more moderate increase in IFN-γ production. On the other hand, the level of IL-4 released from all groups were similar.

Co-expression of ICAM-1 dramatically increases production of β-chemokines by stimulated T cells

Recently we reported that $CD8^+$ effector T cells expand antigen-specific responses *in vivo* through the production of specific chemokines at the peripheral site of infection. Therefore, we analyzed the production of β-chemokines by stimulated CTLs. We analyzed supernatant from the effector cells stimulated *in vitro* for CTL assay and tested them for the release of β-chemokines MIP-1α, MIP-1β, and RANTES. As we had previously observed, we found that DNA immunization with pCEnv induced significantly greater levels of expression of MIP-1α, MIP-1β, and RANTES over those of control vector. Moreover, we observed that co-injection with pCEnv+pCLFA-3 increased the level of β-chemokine production over that of the pCEnv immunized group. Even more significantly, co-immunization with pCEnv+pCICAM-1 resulted in a dramatic enhancement (2-4 fold) of MIP-1α, MIP-1β, and RANTES production over that of the pCEnv immunized group. In contrast, co-administration of pCVCAM-1 did not enhance the level of chemokine expression. These results support that ICAM-1 and LFA-3 provide direct T cell costimulation.

Co-expression of ICAM-1 and CD86 synergistically enhances Ag-specific CTL responses

The B7 (CD80 and CD86) pathway is considered to be a major costimulatory pathway for the delivery of critical second signals to prime and expand T cell responses. These molecules have been examined in the context of DNA vaccines as modulatory agent. In this context, it appears that CD86 molecules play a prominent role in the antigen-specific induction of $CD8^+$ cytotoxic T lymphocytes when delivered as vaccine adjuvants. Co-administration of CD86 cDNA along with DNA immunogens dramatically increased antigen-specific $CD8^+$ CTL response. The effects of ICAM-1 and LFA-3 could be dependent on B7-CD28 signals, or they could represent an alternative synergistic pathway for driving CTL induction *in vivo*. Therefore, we further investigated whether ICAM-1 and LFA-3 molecules when co-expressed with CD86 molecule could synergistically enhance the level of CTL induction. We observed that co-expression of ICAM-1 and CD86 molecules could synergistically enhance antigen-specific CTL response. On the other hand, co-expression of LFA-3 and CD86 molecules did not improve the level of CTL response. These results indicate that ICAM-1/LFA-1 pathways provide T cell costimulatory signals independent of CD86/CD28 pathways, and they may work synergistically to expand T cell responses in vivo.

The level of IFN-γ and β-chemokines MIP-1α, MIP-1β, and RANTES production by stimulated CTLs further support these results indicating that ICAM-1/LFA-1 signals work independent of CD86/CD28 signals and work concordantly to expand T cell responses. When we analyzed supernatant from the effector T cells using the methods described above, we observed that co-administration of LFA-3 and CD86 genes resulted in a dramatically higher level of IFN-γ, MIP-1α, MIP-1β, and RANTES. These results further imply the synergistic nature of ICAM-1 and CD86 in T cell activation.

Discussion

During immune or inflammatory response, lymphocytes traffic to the site of antigen exposure. Adhesion molecules on lymphocytes and endothelial cells play an important role in providing direct cellular contact and directing the migration of the leukocytes. In addition, adhesion molecules play an important role in the binding of T lymphocytes to APCs. ICAM-1 (CD54) is a 90-114 kD molecule which is expressed on endothelial cells, macrophages, and dendritic cells, and binds to LFA-1 and Mac-1. Almost all leukocytes, including T lymphocytes express LFA-1, whereas Mac-1

expression is more restricted to monocytes, macrophages, and granulocytes. LFA-3 (CD58) is a 55-70 kD surface molecule expressed by various cell types including the APCs (Springer, T. A., et al. 1987 *Ann. Rev. Immunol.* 5:223-252, which is incorporated herein by reference). Vascular cell adhesion molecule-1 (VCAM-1) is a 110 kD surface molecule which is expressed on activated endothelial cell and smooth muscle cells (Osborn, L., et al. 1989. *Cell.* 59:1203-1211 which is incorporated herein by reference). VCAM-1 recognizes and binds to very late antigen-4 (VLA-4) which is constitutively expressed on most mononuclear leukocytes, including the eosinophils, lymphocytes, monocytes, and basophils, but is absent on neutophils (Elices, M. J., et al. 1990 *Cell.* 60:577-584, which is incorporated herein by reference). VCAM-1/VLA-4 interaction plays an important role in leukocyte migration and diapedesis.

In this study, we utilized a DNA immunogen model to investigate the roles of these cell surface adhesion molecules in providing stimulatory signals required for T cell activation and expansion. In a two signal T cell activation model, the primary activation signal is mediated by the ligation of antigenic peptide-MHC complexes to T cell receptor. The secondary costimulatory signal is provided through the ligation of CD80/CD86 costimulatory molecules with their receptors (CD28/CTLA-4) present on T cells. Although this two-signal model is conceptually straight-forward and well supported by experimental results, the costimulatory signals provided during T cell activation process may not be restricted only to the B7 (CD80/CD86) molecules. Additional cell surface molecules such as the adhesion molecules on the APCs may also have an important function in providing costimulation, and their roles in providing direct signals to $CD4^+$ and $CD8^+$ T cells are under investigation.

Adhesion molecules are important in leukocyte trafficking, inflammatory cell recruitment, and immune surveillance. Recently, a role for adhesion molecules in T cell activation has been suggested. We investigated the role using a subset of adhesion molecules which all bind to ligands on T cells. We chose three related molecules ICAM-1 (CD54), LFA-3 (CD58), and VCAM-1 (CD106). Utilizing DNA expression cassettes encoding for ICAM-1, LFA-3, and VCAM-1 along with our DNA immunogens, we sought to identify the specific effects of co-expressing adhesion molecules along with antigens. We observed that antigen-specific T cell (both $CD4^+$ and $CD8^+$ T cells)

responses can be enhanced by the co-expression of DNA immunogen and adhesion molecules ICAM-1 and LFA-3. Co-expression of ICAM-1 or LFA-3 molecules along with DNA immunogens resulted in a significant enhancement of Th cell proliferative responses. In addition, co-immunization with pCICAM-1 (and more moderately with pCLFA-3) resulted in a dramatic enhancement of CD8-restricted CTL responses. These observations were further supported by the finding that co-injection with LFA-3 increased the production level of IFN-γ as well as β-chemokines MIP-1α, MIP-1β, and RANTES by stimulated $CD8^+$ T cells. More impressively, co-immunization with ICAM-1 resulted in a more dramatic enhancement of IFN-γ and β-chemokines. It is also important to note that increased cellular contact or juxtaposition of cells alone was not enough to enhance antigen-specific T cell-mediated responses. Even though ICAM-1 and VCAM-1 have similar molecular sizes, the co-injection with VCAM-1 did not have any measurable effect on T cell responses. On the other hand, ICAM-1 co-expression dramatically enhanced the level of both CD4+ and CD8+ T cell responses. These results imply that the T cell stimulatory effects are not inherent to their adhesion properties or the size of the molecules. It is interesting that both CTL driving adhesion molecules which enhanced CTLs (ICAM-1 and LFA-3) are expressed on a variety of APCs. In fact, it may be important that the best CTL inducing adhesion molecule, ICAM-1, is expressed on dendritic cells.

We also compared the enhanced induction of CTLs with that enhanced with CD86 expression. We observed that combining the expression of CD86 molecules with ICAM-1, but not LFA-3 molecules could enhance antigen-specific CTL responses. These results were further supported by significantly enhanced production of IFN-γ as well as β-chemokines MIP-1α, MIP-1β, and RANTES which play important role in immune activation in the periphery. Even though the elucidation of the biological significance of these molecules requires further studies, a recent study found a relationship between chemokines MIP-1β and RANTES and CTL response. Although additional studies could provide more insight into the costimulatory role of these molecules, these results indicate that ICAM-1 molecules can provide T cell costimulatory signals through an independent pathway to CD86, and they may work synergistically to amplify the total level of costimulatory signals provided to T cells.

Overall, these results support that adhesion molecules, ICAM-1 and LFA-3 can provide important costimulatory signals, indicating that the simple two-signal model of T cell activation, although conceptually useful, may be incomplete, and a newer model with multiple sources of costimulation should be further considered and studied. These results also indicate that further studies aimed at utilizing the T cell costimulatory function of other cell surface molecules are warranted.

One important issue with regard to these studies is exactly where are these molecules functioning to enhance the cellular immune response. Recent studies have reported that injection of plasmid DNA can, with low efficiency, transfect resident APC's including macrophages and dendritic cells. These results are further supported by studies using bone-marrow chimeras which illustrate the requirement for bone-marrow derived cells to prime DNA immune responses. It is consistent with the literature that some costimulation observed in our study can occur through transfection and enhanced T cell priming by resident professional APC's.

Along with previous reports, these results support the role for ICAM-1 and LFA-3 in T cell costimulation. It appears that LFA-3 has particular effects on Class II responses while in general, ICAM-1 was a dramatically strong driver of CTL induction and $CD8^+$ effector function as demonstrated by enhanced production of β-chemokines. These results also support a concordant hypothesis for the recruitment and expansion of T cell effectors in the periphery. We recently reported that in addition to their chemoattractant functions, chemokines regulate modulation and expansion of antigen-specific immune responses at the peripheral site. We observed that CD8+ T effector cells control chemokine expression levels while they primed immune responses. Thus, in chemotaxis, chemokines regulate the movement of lymphocytes through a concentration gradient. Moreover, commensurate redistribution of adhesion molecule expression provide direct cell-to-cell contact in directing the lymphocytes to the periphery. In addition, expression of adhesion molecules are modulated by various inflammatory cytokines and chemokines. For instance, IFN-γ and TNF-α have been shown to upregulate ICAM-1 expression on endothelial and muscle cells.

$CD8^+$ effector T cells therefore elaborate chemokines which would recruit more APCs and T cells to the site of inflammation. These T cells would be stimulated by β-

chemokine production to enhance expression of adhesion molecules which could serve to drive IFN-γ production and allow for T cell costimulation. Thus, once at the site of inflammation, these effector CTLs can be further regulated through the expression of specific chemokines and adhesion molecules which would expand the level of effector function. These results further support that end-stage effector T cells in the expansion phase of an antigen-specific immune response could direct their destiny through coordinated expression and release of these molecules.

Example 6: Adhesion and costimulatory molecules induce distinct antigen-specific immune responses and enhance protective immunity against herpes simplex virus-2 in vivo

CD40 ligand and leukocyte function associated proteins (LFA) on T cells interact with CD40 and intercellular adhesion molecules (ICAM) on APC, respectively. We coimmunized with costimulatory molecules CD40 and CD40 ligand, and adhesion molecules LFA-3 and ICAM-1, and then analyzed immune modulatory effects on a gD plasmid vaccine and on protection against lethal challenge with HSV-2. We observed that systemic gD-specific IgG production was significantly enhanced by coinjection with LFA-3. However, little change in IgG production was observed by coinjection with CD40, CD40 ligand and ICAM-1. Furthermore, Th1 type cellular responses were driven by CD40 ligand, whereas both Th1 and Th2 type immune responses were driven by LFA-3. Codelivery with CD40 ligand and LFA-3 also enhanced survival rate from lethal HSV-2 challenge. These studies demonstrate that costimulatory and adhesion molecules have distinct costimulatory pathways and that they can play an important role in generating protective antigen-specific immunity.

The specific roles of costimulatory and adhesion molecules in the induction of antigen-specific immune responses were tested as well as vaccine effect of using costimulatory and adhesion molecule as part of plasmid delivery to drive DNA vaccine induced protective immunity in a mouse HSV-2 challenge model system. We observed that costimulatory and adhesion molecules differentially modulate antigen-specific immune responses. In particular, co-delivery with costimulatory molecule, CD40 ligand

and adhesion molecule, LFA-3 induced significant $CD4^+$ T cell activities in an antigen dependent manner and enhanced survival from lethal HSV-2 challenge.

Materials and methods

Mice - Female 4- to 6-week-old BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.). They were cared for under the guidelines of the National Institutes of Health (Bethesda, Md.) and the University of Pennsylvania IACUC (Philadelphia, Pa.).

Reagents - HSV-2 strain 186 (a kind gift from P. Schaffer, University of Pennsylvania, Philadelphia, Pa.) was propagated in the Vero cell line (American Type Culture Collection, Rockville, Md.). Recombinant HSV-2 gD proteins were used as recombinant antigens in these studies. Human rhabdomyosarcoma (RD) cell line was obtained from ATCC (Rockville, Md.).

Plasmids and DNA Preparation - The DNA vaccine, pAPL-gD2 encoding HSV-2 gD protein was prepared as described in Pachuk, et al. 1998 Current topics Microbiol. Immunol. 226, 79, which is incorporated herein by reference. The cDNA for CD40, CD40 Ligand, LFA-3 and ICAM-1 were cloned into the expression vector pCDNA3 to produce pCDNA3-CD40, pCDNA3-CD40 Ligand, pCDNA3-LFA and pCDNA3-ICAM-1, respectively. Plasmid DNA was produced in bacteria and purified by double banded CsCl preparations.

In vitro expression of CD40 and CD40 ligand gene constructs - Expression of CD40, CD40 Ligand, LFA-3, and ICAM-1 constructs were analyzed by transfecting them into RD cells. Cells were harvested 72 hours after transfection and tested for expression using FACS analysis with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for LFA-3, ICAM-1, CD40 and CD40 Ligand (Pharmingen, San Diego, CA).

DNA inoculation of mice - The quadriceps muscles of BALB/c mice were injected with gD DNA constructs formulated in 100 µl of phosphate-buffered saline and 0.25% bupivacaine-HCl (Sigma, St. Louis, Mo.) via a 28-gauge needle (Becton Dickinson, Franklin Lakes, N.J.). Samples of various chemokine and cytokine gene expression cassettes were mixed with pgD plasmid solution prior to injection.

ELISA - Enzyme-linked immunosorbent assay (ELISA) was performed to determine the relative levels of gD-specific IgG subclasses using anti-murine IgG1 and IgG2a conjugated with HRP (Zymed, San Francisco, CA). The ELISA titers were determined as the reverse of the highest sera dilution showing the same optical density as sera of naive mice.

Chemokines, Th1 and Th2 type cytokines - A 1 ml aliquot containing 6 x 10^6 splenocytes was added to wells of 24 well plates. Then, 1 μg of HSV-2 gD protein/ml was added to each well. After 2 days incubation at 37°C in 5% CO_2, cell supernatants were secured and then used for detecting levels of IL-2, IL-10, IFN-γ, RANTES, MCP-1, and MIP-1α using commercial cytokine kits (Biosource, Intl., Camarillo, Ca. and R&D Systems, Minneapolis, Md.) by adding the extracellular fluids to the cytokine or chemokine-specific ELISA plates.

Intravaginal HSV-2 challenge - Before inoculating the virus, the intravaginal area was swabbed with a cotton tipped applicator (Hardwood Products Company, Guiford, ME) soaked with 0.1 M NaOH solution and then cleaned with dried cotton applicators. Mice were then examined daily to evaluate survival rates.

Statistical analysis - Statistical analysis was done using the paired Student's t test and ANOVA. Values between different immunization groups were compared. p values < 0.05 were considered significant.

Results

CD40, CD40 ligand, LFA-3, and ICAM-1 can be expressed by transfected cells - The genes for CD40, CD40 ligand, LFA-3, and ICAM-1 were individually cloned into the pCDNA3 expression vector. To test whether CD40, CD40 ligand, LFA-3 and ICAM-1 constructs could express their relevant proteins, we transfected them in vitro into the human RD cells. Using FACS analysis we observed that transfection of CD40, CD40 ligand, LFA-3, and ICAM-1 expression cassettes resulted in specific expression of CD40, CD40 ligand, LFA-3 and ICAM-1, respectively. RD cells were transfected with pCDNA3 (control) or pCDNA3 expressing CD40, CD40 Ligand, LFA-3 and ICAM-1. Three days following transfection the cells were removed from the plates and were analyzed by FACS analysis using α-CD40, α-CD40 ligand, α-LFA-3, α-ICAM-1 antibodies to detect expression of the transfected gene product.

LFA-3 enhances systemic IgG response - To determine if coinjection of gD genetic vaccines with CD40, CD40 ligand, LFA-3 and ICAM-1 expression vectors might influence systemic IgG responses against gD, one hundred dilution of sera after the DNA inoculation was tested in ELISA. Each group of mice (n=8) was immunized twice with gD DNA vaccines (60 μg) plus costimulatory molecule genes (40 μg). Mice were bled 2 weeks after the second DNA injection and then sera were diluted to 1:100 for reaction with gD. Coinjection with gD DNA vaccine (60 μg per mouse) plus CD40 or CD40 ligand plasmid DNAs (40 μg per mouse) had no significant effect on overall IgG levels. Equally pooled sera per group were serially diluted to determine ELISA titer. ELISA titers of equally pooled 2 week post second immunization were also determined as 6,400 (CD40), 6,400 (CD40 ligand), and 6,400 (gD DNA vaccine alone). The similar result was also observed when we tested sera obtained 1 month after coinjecting once with gD DNA vaccine (10 μg per mouse) plus these costimulatory molecules (40 μg per mouse).

Groups of mice (n=8) were immunized with gD DNA vaccines (60 μg) plus LFA-3 adhesion molecule genes (40 μg) at 0 and 2 weeks. Mice were bled bi-weekly and then sera were diluted to 1:100 for reaction with gD. Coinjection with LFA-3 cDNA enhanced systemic IgG responses significantly higher than gD DNA vaccine alone while little change was observed by coinjecting ICAM-1 cDNA. Equally pooled sera per group were serially diluted to determine ELISA titer. Optical density was measured at 405 nm. Values and bars represent mean (n=8) and the standard deviation. The ELISA titers were determined to be the reverse of the highest dilution showing the same optical density as sera of naïve mice. ELISA titers of equally pooled 2 week post second immunization were also determined as 25,600 (LFA-3), 6,400 (ICAM-1), and 6,400 (gD DNA vaccine alone).

CD40 ligand and LFA-3 influence IgG isotype pattern. IgG subclasses give an indication of the Th1 vs Th2 nature of the induced immune responses. We analyzed the IgG subclasses induced by the coinjections with CD40, CD40 ligand, LFA-3 and ICAM.-1. Levels of gD-specific IgG isotypes in mice immunized with DNA vectors were measured. Each group of mice (n=8) was immunized twice with gD DNA vaccines (60 μg) plus either costimulatory molecule genes (40 μg) or adhesion molecule genes (40 (g). Mice were bled 2 weeks after the second DNA injection and then equally pooled

sera per group were diluted to 1:100 for reaction with gD. Optical density was measured at 405 nm. Coinjection with CD40 ligand genes increased the relative production of gD-specific IgG2a to IgG1, whereas coinjection with CD40 genes showed the similar IgG isotype pattern to gD DNA vaccine alone. This shift in IgG production illustrates that a more Th1 type response is induced by coinjection with only CD40 ligand cDNA. However, coinjection with LFA-3 increased both IgG1 and IgG2a isotypes significantly higher than gD DNA vaccine alone or ICAM-1 coinjection. This increase indicates that both Th1 and Th2 type responses are induced by coinjection with LFA-3 cDNA

CD40 ligand and LFA-3 enhance Th cell proliferation responses - T helper cells play an important role in eliciting both humoral and cellular immune responses via expansion of Ag stimulated B cells and expansion of $CD8^+$ T cells, respectively. As a specific indicator of CD4 activation T cell proliferation was examined. It is important to measure proliferation levels of T cells obtained after coimmunization with cytokine genes when stimulated in vitro with a specific antigen. The gD-2 protein (1 and 5 μg/ml) was used for antigen specific stimulation of T cells. For a positive control, 5 μg/ml PHA was used as a polyclonal stimulator. A low background level of Th cell proliferation was observed in negative controls. However, gD DNA vaccination induced Th cell proliferation responses much higher than negative controls. When coinjected with CD40 ligand and LFA-3 cDNAs, Th cell proliferation levels were further boosted. However, little increase in Th cell responses was detected in animals coinjected with CD40 and ICAM-1 cDNAs. This tendency was observed over the two different gD antigen concentrations tested, reflecting that this effect is CD40 ligand and LFA-3-mediated. The gD plasmid vaccination does not result in CTL responses due to a lack of CTL epitope in the Balb/c background. However, to evaluate cellular effects in more detail we next examine cytokine production profiles.

CD40 ligand and LFA-3 influence production of Th1 and Th2 type cytokines. Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4, IL-5 and IL-10) have been a mainstay in our understanding of the polarization of immune responses. Th1 immune responses are thought to drive induction of cellular immunity, whereas Th2 immune responses preferentially drive humoral immunity. Thus, we examined whether gD DNA vaccination with and without costimulatory molecules induces Th1 or Th2 immune

responses. Production levels of IL-2, IL-10, IFN-γ, RANTES, MIP-1α and MCP-1 from splenocytes in mice coimmunized with either costimulatory molecules or adhesion molecules were measured. Each group of mice (n=2) was immunized with gD DNA vaccines (60 μg) plus either costimulatory molecule genes or adhesion molecule genes (40 μg) at 0 and 2 weeks. Two weeks after the last DNA injection, two mice were sacrificed and spleen cells were pooled. Splenocytes were stimulated with 1μg/ml gD-2 proteins for 2 days. IL-2, and IFN-γ productions were significantly enhanced by coinjection with CD40 ligand cDNA while IL-10 production was reduced by this coinjection. However, coinjection with CD40 plus gD genes had slightly increasing effect on IFN-γ in this assay. However, IL-2, IL-10 and IFN-γ productions were all enhanced by coinjection with LFA-3 cDNA significantly higher than gD DNA vaccine, whereas coinjection with ICAM-1 plus gD genes had slightly increasing effect in this assay. This supports that CD40 ligand drives immune responses towards Th1 phenotype while LFA-3 influences both Th1 and Th2 immune phenotypes in vivo.

CD40 ligand and LFA-3 influence production of β chemokines - Beta chemokines (CC type) including RANTES (regulated on activation, normal T cell expressed and secreted), MIP (macrophage inflammatory protein)-1α, and MCP (monocyte chemotactic protein (MCP)-1 chemoattact particularly monocytic phagocytes, and activate T cells, basophils, eosinophils, and mononuclear phagocyte as well as a variety of other soluble immune modulators. As compared to MCP-1, RANTES and MIP-1α were also reported to be a major HIV suppressive factor. These molecules are thought to be important in modulating inflammatory immune responses. However, their direct role in infectious diseases is under investigation. The relationship of CD40, CD40 ligand, LFA-3 and ICAM-1 molecules as a vaccine adjuvant to chemokine production in vivo is unknown. We investigated the levels of chemokines (RANTES, MCP-1, and MIP-1α) induced by coinjection with gD DNA vaccine plus CD40 ligand, CD40, LFA-3 and ICAM-1 cDNAs. gD DNA vaccine alone enhanced production of RANTES, MCP-1 and MIP-1α in an antigen specific manner. Furthermore, coinjection with CD40 ligand cDNA enhanced RANTES and MIP-1α production significantly higher than gD DNA vaccine alone. In contrast, MCP-1 production was unaffected by CD40 ligand coinjection. However, coinjection with CD40 molecules showed slightly increasing

effect on theβ(chemokine production. Data showed that coinjection with LFA-3 cDNA enhanced RANTES and MIP-1α production significantly higher than gD DNA vaccine alone. Similarly, production of RANTES and MIP-1α was enhanced by ICAM-1coinjection. In contrast, MCP-1 production was inhibited by ICAM-1 coinjection. This modulation supports that costimulatory and adhesion molecules can have specific effects on production of individual members of the β chemokine family.

CD40 ligand and LFA-3 enhance protection from intravaginal (i.vag.) HSV challenge. A lethal dose $(LD)_{50}$ of HSV-2 (186) was previously measured. To determine if using CD40, CD40 ligand, LFA-3 and ICAM-1 cDNAs as a molecular adjuvant in gD genetic vaccination could influence protection from HSV-2 challenge, mice were immunized with both DNA vaccines and the individual costimulatory and adhesion molecule cDNAs, and then challenged i.vag. with 4 LD_{50} of HSV-2. Intravaginal infection route was chosen as HSV-2 infects mucocutaneously and causes urogenital infections. Survival rates of mice immunized twice with gD DNA vaccines plus costimulatory or adhesion molecule genes were measured. Each group of mice (n=10) was immunized once with gD DNA vaccines (10 μg) plus costimulatory or adhesion molecule genes (40 μg). Four weeks after the DNA immunization, mice were challenged i.vag. with 4 LD_{50} of HSV-2 strain 186 (1.4×10^4 pfu). When mice were immunized with gD DNA vaccine, 60% of survival was noted, but all naive mice died within 13 days following viral challenge. However, coinjection with CD40 ligand increased survival rate to 100%, a 40% enhancement of protection rate, whereas coinjection with CD40 cDNA showed minimal protective effects, as compared to gD DNA vaccine alone. Furthermore, coinjection with LFA-3 cDNA increased survival of mice to 90%. However, coinjection with ICAM-1 cDNA showed slightly better effects on protection from HSV-2 infection.

Discussion

During antigen presentation, costimulatory molecules of APC are important for the initiation and differentiation of T cell responses. In particular, the CD40L-CD40 interaction induces B7 and IL-12 expression from APC. IL-12 also enhances CD40 ligand expression from T cells , whereas IFN-γ inhibits CD40 ligand expression, indicating that there might be an auto-regulatory mechanism for induction of CD40